Calcium (Ca$^{2+}$) is a universal signal that controls a variety of cell functions of major importance in health and disease. Stimulation of specific phospholipase C (PLC)-coupled receptors generates spatio-temporal Ca$^{2+}$ signals pivotal for control of cellular responses, such as gene transcription, contraction, secretion, migration, proliferation, and apoptosis.$^{1,2}$ These receptor-activated Ca$^{2+}$ entry pathways comprise the following: (1) store-operated Ca$^{2+}$ entry (SOCE) channels activated by inositol 1,4,5-trisphosphate (IP$_3$)-mediated depletion of Ca$^{2+}$ from the endoplasmic reticulum (ER) stores$^{1}$ and (2) store-independent Ca$^{2+}$ entry (non-SOCE) channels activated by largely unknown mechanisms that do not depend on the state of filling of ER stores.$^4$ Whether SOCE and store-independent Ca$^{2+}$ channels are concomitantly activated by the same agonist or whether specific agonists selectively activate specific Ca$^{2+}$ channels remain unknown.

Rationale: Through largely unknown mechanisms, Ca$^{2+}$ signaling plays important roles in vascular smooth muscle cell (VSMC) remodeling. Orai1-encoded store-operated Ca$^{2+}$ entry has recently emerged as an important player in VSMC remodeling. However, the role of the exclusively mammalian Orai3 protein in native VSMC Ca$^{2+}$ entry pathways, its upregulation during VSMC remodeling, and its contribution to neointima formation remain unknown.

Objective: The goal of this study was to determine the agonist-evoked Ca$^{2+}$ entry pathway contributed by Orai3; Orai3 potential upregulation and role during neointima formation after balloon injury of rat carotid arteries.

Methods and Results: Ca$^{2+}$ imaging and patch-clamp recordings showed that although the platelet-derived growth factor activates the canonical Ca$^{2+}$ release-activated Ca$^{2+}$ channels via store depletion in VSMC, the pathophysiologically agonist thrombin activates a distinct Ca$^{2+}$-selective channel contributed by Orai1, Orai3, and stromal interacting molecule1 in the same cells. Unexpectedly, Ca$^{2+}$ store depletion is not required for activation of Orai1/3 channel by thrombin. Rather, the signal for Orai1/3 channel activation is cytosolic leukotrieneC$_4$ produced downstream thrombin receptor stimulation through the catalytic activity of leukotrieneC$_4$ synthase. Importantly, Orai3 is upregulated in an animal model of VSMC neointimal remodeling, and in vivo Orai3 knockdown inhibits neointima formation.

Conclusions: These results demonstrate that distinct native Ca$^{2+}$-selective Orai channels are activated by different agonists/pathways and uncover a mechanism whereby leukotrieneC$_4$ acts through hitherto unknown intracrine mode to elicit store-independent Ca$^{2+}$ signaling that promotes vascular occlusive disease. Orai3 and Orai3-containing channels provide novel targets for control of VSMC remodeling during vascular injury or disease. (Circ Res. 2013;112:1013-1025.)

Key Words: calcium signaling ■ ion channel ■ neointima formation ■ Orai1 ■ Orai3 ■ STIM1 ■ vascular smooth muscle
SOCE channels mediate the highly Ca$^{2+}$-selective, Ca$^{2+}$-release-activated Ca$^{2+}$ (CRAC) current. The past several years have brought about remarkable advances in our understanding of the SOCE pathway with the identification of stromal interacting molecule1 (STIM1) as the ER Ca$^{2+}$ sensor and Orai1 as the pore-forming unit of CRAC channels at the plasma membrane (PM). Depletion of ER Ca$^{2+}$ stores causes oligomerization of STIM1 and its accumulation in punctuate structures within regions of close contacts (10–25 nm) between the ER and PM. Direct STIM1–Orai1 interaction involving the binding of a minimal C-terminal 100 amino acid domain of STIM1 (called STIM Orai activating region) to the C-and N-termini of Orai1 activates local Ca$^{2+}$ entry through CRAC channels. Currents measured in cell lines, such as rat basophilic leukemia mast cells and Jurkat T cells: In rat basophilic leukemia cells, for example, CRAC current density is 1 to 2 pA/pF at −100 mV. The paucity of electrophysiological recordings of native store-operated Ca$^{2+}$-selective conductances in response to physiological agonists in primary cell types, such as vascular smooth muscle cells (VSMCs), is largely attributable to the tiny single-channel conductance of CRAC channels (≈15 femto-Siemens) coupled to the low-expression levels of STIM1 and Orai1 in these primary cells; CRAC current density is 0.1 to 0.3 pA/pF at −100 mV in cultured VSMCs.

Although it is generally accepted that Orai1 homologs, Orai2, and Orai3 mediate SOCE and CRAC currents when coexpressed with STIM1 in HEK293 cells, a very interesting finding from a large number of mammalian cell types and tissues studied so far is that native SOCE is exclusively mediated by Orai1, despite concomitant and abundant expression of Orai2 and Orai3 proteins in these systems; the exception is an instance where Orai3 encodes SOCE in breast cancer cell lines that expresses estrogen receptors. This raises the interesting possibility that homo- and hetero-multimers contributed by Orai2 and Orai3 might encode Ca$^{2+}$-selective channels distinct from CRAC that are activated by alternative store-independent mechanisms that would enhance the diversity of cellular Ca$^{2+}$-selective conductances. In fact, although a great deal of attention has been focused on the SOCE pathway, there is increasing evidence for several non-SOCE pathways in various cell types. The likely signal for activation of store-independent Ca$^{2+}$ entry pathways is second messengers-generated downstream PLC activity. However, (1) the molecular identity, (2) the exact mechanisms of second messenger action, (3) the specific agonist requirement, and (4) the ionic currents mediating these pathways, and (5) the physiological and pathophysiological functions controlled by these pathways remain largely obscure. One exception is the store-independent Ca$^{2+}$-selective channel mediating the arachidonic acid (AA)-regulated Ca$^{2+}$ (ARC) current. ARCs have been shown to be activated by AA in HEK293 cells and to be contributed by both Orai1 and Orai3 subunits and regulated by a specific pool of PM-resident STIM1. Members of the transient receptor potential canonical (TRPC) family are either activated by diacylglycerol-produced downstream of PLC (TRPC3/6/7) or by store depletion through STIM1 (TRPC1/3/4/5/6). However, TRPCs are nonselective cation channels carrying mainly Na$^{+}$ ions and have protein structures that are different from Orai channels, suggesting that TRPCs’ and Orai’s contributions to native Ca$^{2+}$ entry pathways and cell functions are likely nonredundant.

VSMCs are one of the major cell types in blood vessels and play major roles in vessel integrity and control of blood pressure. Unlike cardiac and skeletal muscles that are terminally differentiated, VSMCs are plastic in nature and retain the ability to switch in vivo from a contractile excitable phenotype to a proliferative migratory nonexcitable phenotype (also called synthetic), a condition that can be recapitulated in culture. This VSMC phenotype modulation is essential for vascular development, angiogenesis, and repair. However, its dysfunction contributes to vascular diseases, such as atherosclerosis, hypertension, restenosis, and leiomyosarcomas. VSMC phenotypic modulation is characterized by downregulation of L-type Ca$^{2+}$ channels and upregulation of STIM1 and Orai1. STIM1 and Orai1 were shown to be required for VSMC phenotypic switch in vitro and neointima formation in animal models of vascular injury, supporting a role for agonist-activated Ca$^{2+}$ entry pathways in driving VSMC phenotypic modulation during disease. Indeed, mitogenic migratory and inflammatory agonists, such as the platelet-derived growth factor (PDGF) and thrombin, are major contributors to vascular remodeling that are heavily produced and see the expression of their receptors increased during vascular injury.

In this study, we apply improved low-noise high-resistance (>16 GΩ) whole-cell patch-clamp recordings, amplify and measure reliable, tiny, highly Ca$^{2+}$-selective SOCE and non-SOCE currents from primary synthetic VSMCs in response to PDGF and thrombin. We show that PDGF and thrombin, established VSMC pathophysiological agonists, activate distinct Ca$^{2+}$-selective channels involving distinct mechanisms; whereas PDGF activates classical store-dependent CRAC currents mediated by Orai1 and thrombin activates a store-independent current encoded by both Orai1 and Orai3 that...
requires intracellular leukotrieneC\(_4\) (LTC\(_4\))-produced down- 
stream receptor activation. Finally, VSMC Orai3 and LTC\(_4\)- 
activated currents are upregulated in vivo in an animal model 
of carotid vessel injury, and Orai3 knockdown using specific 
shRNA-encoding lentiviruses inhibits these currents as well 
as VSMC remodeling and neointima formation.

**Methods**

List of reagents used throughout the study methods and detailed 
experimental procedures on VSMC dispersion and culture, cell 
transfections, Ca\(^{2+}\) measurements, patch-clamp electrophysiology, 
Förster resonance energy transfer microscopy, and balloon injury of 
rat carotid arteries are provided in the Online Data Supplement.

**Results**

**Thrombin-Activated Ca\(^{2+}\) Entry and Currents Are 
Distinct From SOCE and CRAC Currents**

Previous studies from our group showed that the proprolif-
erative agonist PDGF activates Ca\(^{2+}\) entry through classical 
SOCE in rat synthetic VSMCs.\(^{30}\) Interestingly, Fura2 Ca\(^{2+}\)- 
imaging protocols with agonist stimulation in a nominally 

![Figure 1. Thrombin-activated Ca\(^{2+}\) entry and currents are additive to store-operated calcium entry and calcium release-
activated calcium (CRAC) and are store independent. A, Ca\(^{2+}\) imaging experiments showing additivity between thrombin-
(100 nmol/L) and platelet-derived growth factor (PDGF)-activated (100 ng/mL) Ca\(^{2+}\) entry pathways. B, Whole-cell patch-clamp 
electrophysiology shows additivity of CRAC currents (activated by dialysis of 20 mmol/L BAPTA through the patch pipette for 6 min) 
and thrombin-activated currents (Na\(^+\) current/voltage [I/V] depicted in C and statistics in D). E, Endoplasmic reticulum (ER)-Ca\(^{2+}\)- 
levels were measured using the ER-targeted Förster resonance energy transfer (FRET) sensor Cameleon D1ER, before and after 
stimulation with maximal concentrations of thapsigargin (TG, 4 \(\mu\)mol/L; n=12) and thrombin (Th, 500 nmol/L; n=9); only thapsigargin 
caused a significant decrease in ER Ca\(^{2+}\) levels. Whole-cell patch-clamp electrophysiology showing the development of PDGF-
activated CRAC currents with typical depotentiation in divalent-free (DVF) solutions (PDGF, 100 ng/mL; F). Heparin (3 mg/mL) 
dialysis through the patch pipette for 6 minutes completely abrogated PDGF-activated CRAC (G, H). Heparin dialysis failed to inhibit 
the development of thrombin-activated currents (I, K). Na\(^+\) I/V relationships taken from traces (F, G, I) indicated by the color-coded 
esterisks are depicted in J. CFP indicates cyan fluorescent protein.
<table>
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<th>Experiment</th>
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<th>$[\text{Ca}^{2+}]$, pA/pF</th>
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<th>$[\text{Na}^{+}]$, pA/pF</th>
<th>n</th>
<th>$P$ Value</th>
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(Continued)
Ca\textsuperscript{2+}-free solution followed by restoration of Ca\textsuperscript{2+} (2 mmol/L) to the extracellular milieu revealed that thrombin (at maximal concentrations, 100 mmol/L) activates a Ca\textsuperscript{2+} entry pathway in primary synthetic rat aortic VSMCs that is additive to the Ca\textsuperscript{2+} entry activated by PDGF (100 ng/mL; Figure 1a), suggesting that these 2 Ca\textsuperscript{2+} entry pathways are distinct. Whole-cell patch-clamp recordings using a pipette solution containing 20 mmol/L of the chelator BAPTA (to cause maximal store depletion) demonstrated the activation of an inwardly rectifying Ca\textsuperscript{2+}-selective CRAC current (sampled at −100 mV; Figure 1b). Subsequent addition of thrombin (100 mmol/L) to the same cells consistently activated an additional current (Figure 1b), suggesting that thrombin mediates Ca\textsuperscript{2+} entry through a CRAC-independent pathway. CRAC and thrombin-activated currents were recorded in Ca\textsuperscript{2+}-containing (20 mmol/L) bath solutions and amplified in divalent-free (DVF; Na\textsuperscript{+} is the charge carrier) solutions. Both these currents have reversal potentials of around +60 mV (Online Table I). Figure 1c shows the current/voltage (I/V) relationships of CRAC and thrombin-activated currents and their additivity. Positions on traces where I/V curves for currents are taken in this figure and all subsequent figures are indicated by the color-coded signs (eg, asterisks). Statistics on whole-cell current densities measured in DVF solutions on store depletion (first) and subsequent addition of thrombin (second) are shown in Figure 1d. Statistical analyses of patch-clamp data (mean±range, n) and probability values for comparisons done from this figure and all subsequent figures are reported in Table 1.

### Thrombin-Activated Ca\textsuperscript{2+} Entry and Currents Are Store Independent

Because thrombin-activated Ca\textsuperscript{2+} entry and membrane currents are additive to SOCE and CRAC currents activated by either passive store depletion or physiologically by PDGF, 2 following major questions arise: (1) does thrombin, by virtue of activating PLC\textbeta and producing IP\textsubscript{3}, cause store depletion? and (2) is store depletion required for activation of Ca\textsuperscript{2+} entry and currents in response to thrombin? Surprisingly, using 3 different approaches, detailed below, we found that thrombin does not cause sustained store depletion, only reversible Ca\textsuperscript{2+} release, and the Ca\textsuperscript{2+} entry and currents it activates do not require store depletion:

1. Ca\textsuperscript{2+} imaging experiments with sequential addition of PDGF followed by thrombin and vice versa at maximally saturating concentrations (500 mmol/L thrombin and 500 ng/mL PDGF) in nominally Ca\textsuperscript{2+}-free solutions showed that while PDGF caused store depletion, thrombin did not. Using the following protocol, PDGF was added first in nominally Ca\textsuperscript{2+} free followed by extracellular Ca\textsuperscript{2+} restoration for 6 minutes (to prevent excessive Ca\textsuperscript{2+} leak from ER) in the continuous presence of PDGF (to maintain IP\textsubscript{3} production), followed by thrombin addition in nominally Ca\textsuperscript{2+} free, thrombin failed to cause Ca\textsuperscript{2+} release. However, when thrombin was added first followed by PDGF using the same protocol, PDGF, though added second, caused comparable Ca\textsuperscript{2+} release to when it was added first (Online Figure Ia–d).

2. The use of the ER-targeted Ca\textsuperscript{2+} dye Cameleon D1ER coupled for Förster resonance energy transfer fluorescence...
microscopy showed that although thapsigargin was very effective at causing ER Ca\(^{2+}\) store depletion, maximal concentrations of thrombin had only a small and transient effect (Figure 1e). Similar experiments showed that PDGF also caused store depletion but at a faster rate and to a lesser extent than thapsigargin (Online Figure Ie).

3. Whole-cell patch-clamp recordings using a pipette solution where free Ca\(^{2+}\) was buffered to 150 nmol/L with BAPTA showed that PDGF-activated a Ca\(^{2+}\)-selective current reminiscent of CRAC that showed the typical depotentiation in DVF solutions\(^{34}\) (Figure 1f). Inclusion of heparin in the patch pipette, destined to inhibit IP\(_3\) receptors, completely abrogated CRAC currents activated by PDGF as would be expected for a store-dependent current (Figure 1g). However, thrombin-activated currents did not depotentiate in DVF solutions and were normally activated in the presence of heparin (Figure 1i). The I/V curves taken indicated in traces by color-coded asterisks...
are represented in Figure 1j and statistics for PDGF and thrombin are shown in Figure 1h and 1k, respectively. Statistical analyses of patch-clamp data from each figure (mean±range, n) and probability values for group comparisons with control are reported in Table 1.

Thrombin Activates a Ca²⁺-Selective Entry Pathway Mediated by STIM1, Orai1, and Orai3

To determine the molecular identity of thrombin-activated Ca²⁺ entry pathway, we used an unbiased molecular knockdown approach targeting all Orai and TRPC isoforms expressed in synthetic VSMCs. We used the following: (1) infection with specific short hairpin (shRNA)-encoding lentivirus; (2) transfection with specific small interference RNA (siRNA) sequences (Online Table III); and (3) transfection with dominant negative Orai constructs (Figure 2d, image 1) showed that thrombin-mediated Ca²⁺ entry requires STIM1, Orai1, and Orai3, but was independent of Orai2, and the 3 TRPC1/4/6 isomers found expressed in rat synthetic VSMCs²⁶ (Figure 2b–2d). Knockdown of STIM1, Orai1, and Orai3 is shown in Figure 2a, whereas knockdown of Orai2 is documented in Online Figure IIa. Statistical analyses on the extent of Ca²⁺ entry is shown in Figure 2d. Please note throughout that the representative Ca²⁺ imaging traces represent averages from several cells on the same coverslip as indicated by n. For statistical analysis, the numbers between parentheses next to each column of bar graphs (x,y) represent x=number of independent runs and y=total number of cells from all these runs. All probability values for comparisons are listed in Online Table II. The unexpected involvement of Orai3 in this pathway prompted us to use yet an additional siRNA sequence against Orai3 and demonstrate that Orai3 protein knockdown inhibits thrombin-activated Ca²⁺ entry without effecting Orai1 and STIM1 protein expression (Online Figure IIb–d).

Thrombin-Activated Ca²⁺ Entry and Currents Require Cytosolic LTC₄

Because store depletion is not required for thrombin-activated Ca²⁺ entry and currents, we systematically evaluated the role of second messengers-produced downstream thrombin receptor in the activation of this pathway. IP₃ dialysis through the patch pipette exclusively activated CRAC currents recognized by their pharmacology and their depotentiation in DVF solutions and addition of a diacylglycerol analog (1-oleoyl-2-acetyl-sn-glycerol; OAG) to the bath solution activated by guest on January 1, 2018 http://circres.ahajournals.org/ Downloaded from
failed to activate whole-cell currents when dialyzed through the patch pipette, whereas subsequent addition of thrombin to the same cells consistently activated Ca\(^{2+}\)-selective currents (Online Figure IIIa–IIIc). Furthermore, addition of LTC\(_4\) to the bath solution did not activate any current, suggesting that LTC\(_4\) acts from the inside and not via its specific PM G-protein–coupled receptors (Online Figure IIId, IIIe). Stimulation of VSMCs with thrombin lead to an increase of LTC\(_4\) production after either 5 or 15 minutes in the presence of thrombin as measured using competitive enzyme-linked immunosorbent assay (Figure 3d; probability values for comparisons are provided in Online Table II). Molecular knockdown of LTC\(_4\)S with either siRNA transfection (with 2 independent siRNA sequences) or shRNA infection (encoding a third sequence) reduced LTC\(_4\)S protein expression (Figure 3e) and inhibited thrombin-activated Ca\(^{2+}\) entry (Figure 3f and 3g) and membrane currents (Online Figure IIIh–IIIk for siRNA and III l–o for shRNA). Control experiments showed that SOCE activated by thapsigargin was insensitive to LTC\(_4\)S knockdown (Online Figure IIIf and IIIg). Significantly, currents activated by direct introduction of LTC\(_4\) into cells were insensitive to LTC\(_4\)S protein knockdown (Figure 3h–3k).

**LTC\(_4\)**-Regulated Ca\(^{2+}\) (LRC) Currents Require STIM1, Orai1, and Orai3

Knockdown experiments using shRNA-encoding lentivirus es showed that STIM1 was required for current activation by direct application of LTC\(_4\) in the patch pipette (Figure 4a–4d), arguing that STIM1 is downstream of LTC\(_4\) action. Control experiments demonstrating that STIM1 knockdown also abrogates CRAC currents in the same cells are shown (Online Figure IV). We showed that Orai1 and Orai3 are both required for thrombin-activated Ca\(^{2+}\) entry (Figure 4). Here, we show that LTC\(_4\) delivered through the patch pipette into VSMCs also requires both Orai1 (Figure 5a–5d) and Orai3 (Figure 5i–5l) as demonstrated with knockdown and overexpression experiments using shRNA-encoding lentiviruses; these results further strengthen the idea that LTC\(_4\) and thrombin activate the same Ca\(^{2+}\) entry pathway in VSMC. Control experiments showed that, in the same cells, store-dependent CRAC currents require Orai1 (Online Figure Va–Vd), but not Orai3 (Online Figure Ve–Vh). Infection with shRNA-encoding lentiviruses against Orai3 (shOrai3) caused downregulation of Orai3 protein levels with no effect on its closest homolog, Orai1 (Figure 5e–5h), thus establishing the specificity of shOrai3 for subsequent in vivo studies.

**Orai3 and LRC Currents Are Upregulated in Medial and Neointima VSMC After Vascular Injury**

Cultured synthetic VSMCs reminiscent of vascular occlusive disease used so far in this study have upregulated protein levels of Orai3 by comparison with quiescent freshly isolated VSMCs that are reminiscent of healthy vessels (Online Figure Vla; please note that β-actin is also known to be upregulated in proliferative VSMCs in vitro and in vivo). Given the established importance of thrombin in vascular injury and vascular occlusive disease\(^{11,13,36}\) and the specific involvement of

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**Figure 4. Stromal interacting molecule 1 (STIM1) is required for leukotriene C\(_4\) (LTC\(_4\))-activated currents.** Whole-cell patch-clamp recordings of LTC\(_4\)-activated currents (A–D) in vascular smooth muscle cells infected with lentiviral vectors encoding either control shRNA against luciferase (shLuc) or STIM1 shRNA (shSTIM1). Depletion of STIM1 completely abrogated currents activated by inclusion of LTC\(_4\) in the patch pipette (B). Na\(^+\) current/voltage (I/V) relationships are shown for LTC\(_4\)-activated currents (C). Statistics on current data are shown in D. Na\(^+\) I/V relationships are taken from current traces indicated by the color-coded asterisks. DVF indicates divalent-free.

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The use of pharmacological reagents targeting the downstream AA metabolism pathway ruled out the requirement of cyclooxygenases (COX1/2) and leukotriene\(_A_4\) hydrolase but suggested requirement of leukotriene\(_C_4\) synthase (LTC\(_4\)S) in thrombin-mediated Ca\(^{2+}\) entry (not shown). Given the molecular similarity between the thrombin-activated Ca\(^{2+}\) entry and arachidonic acid-regulated Ca\(^{2+}\) channels (dependence on STIM1, Orai1, and Orai3), we applied exogenous arachidonic acid (AA; 8 µmol/L) to VSMCs which consistently activated a Ca\(^{2+}\)-selective current that did not depotentiate in DVF solutions and was not additive to currents activated by thrombin (Figure 2e–2g). The addition of thrombin first followed by AA confirmed this lack of additivity (Online Figure Ie–Ig).

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We tested whether introduction of LTC\(_4\) directly into the cytosol through the patch pipette could activate a current reminiscent of thrombin and AA-activated currents. We used a concentration of LTC\(_4\) (100 nmol/L) shown to be physiologically relevant. The LTC\(_4\) dialyzed through the patch pipette activated a Ca\(^{2+}\)-selective current in VSMCs under Ca\(^{2+}\)-containing and DVF solutions, and this current had a reversal potential of +60 mV (Online Table I), did not depotentiate in DVF solutions, and more importantly, was not additive to thrombin-activated currents (Figure 3a–3c). However, a closer leukotriene, LTB\(_4\),...
Orai3 in the thrombin-activated Ca\(^{2+}\) entry pathway, we tested whether Orai3 is also upregulated in vivo in a model of vascular remodeling and neointima formation after balloon injury of rat carotids. The validated shOrai3 lentiviruses, along with lentiviruses encoding nontargeting control shRNA (shNT), were used in vivo in this injury model to determine whether preventing Orai3 upregulation after injury could have inhibitory effects on vascular remodeling and neointima formation. ShOrai3 achieved significant Orai3 knockdown in cultured VSMCs (Figure 5g and 5h), and infection with shOrai3 and shNT lentiviruses led to essentially 100% infection of VSMCs in vitro as visualized using green fluorescent protein encoded by the lentiviruses (Online Figure VIb).

Injury of rat left carotid arteries leads to the apparition of LTC4-regulated Ca\(^{2+}\) (LRC) currents in medial and neointimal VSMC acutely isolated from injured arteries; medial VSMC from noninjured arteries showed no significant LRC currents on dialysis of LTC4 in the cells (Figure 6a–6c). The I/V relationships of whole-cell LRC currents from these different types of cells are shown in Figure 6d, and summary of data is shown in Figure 6e; statistical analyses of data (mean±range; n) are depicted in Table 1. Similarly, we found that CRAC currents, activated by store depletion with dialysis of 20 mmol/L BAPTA through the patch pipette, were evident in medial and neointimal VSMC acutely isolated from injured carotid arteries, whereas medial VSMC from noninjured vessels showed no detectable CRAC currents (Online Figure VII). These results are consistent with previous data reporting upregulation of Orai1 and SOCE on vascular injury.16,29,30

Injury of left rat carotid arteries also caused a significant upregulation of Orai3 proteins in lysates of medial and neointimal VSMCs on day 14 postinjury (Figure 6f) as well as that of Orai1 proteins as previously shown.29 Efficient vessel infection by shOrai3 or shNT-encoding lentiviruses was documented by the expression of lentivirus-encoded green fluorescent protein encoded by the lentiviruses (Online Figure VIIb).

**Orai3 Is Required for LRC Currents and Neointima Formation In Vivo**

Survival surgery involving transduction of injured carotid arteries of anesthetized animals with shOrai3 lentiviruses caused a significant attenuation of Orai3 protein upregulation on day 14 postinjury with no significant effect on Orai1 expression (Figure 6f–6h) by comparison with control shNT. This in vivo Orai3 knockdown corresponded functionally to a decrease in whole-cell LRC current densities in medial (by

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**Figure 5. Orai1 and Orai3 are required for leukotrieneC\(_4\) (LTC\(_4\))-regulated Ca\(^{2+}\) (LRC) currents.** Whole-cell patch-clamp electrophysiology in vascular smooth muscle cells (VSMCs) infected with lentivirus carrying either shRNA against luciferase (shLuc), or shRNA targeting Orai1 (shOrai1). Orai1 knockdown completely abrogated LTC\(_4\)-activated Na\(^+\) currents (B) as compared with control (A). Na\(^+\) current/voltage (I/V) relationships (C) confirm the requirement of Orai1 for LTC\(_4\)-activated currents in VSMCs. Statistical analysis is shown in D. Representative Western blots showing that shRNA targeting Orai3 does not affect Orai1 protein levels (E, F), while significantly abrogating Orai3 protein expression (G, H). Whole-cell patch-clamp electrophysiology in VSMCs infected with lentivirus-encoding either nontargeting control shRNA (shNT) or shRNA targeting Orai3 (shOrai3). Orai3 knockdown completely abrogated LTC\(_4\)-activated currents (J) as compared with control (I). The I/V relationships are shown in K. Statistical analysis of patch-clamp data is shown in L. Values for current densities represented as mean±range and number of independent recordings for shRNA Control, shRNA Orai1, and shRNA Orai3 are reported in Table 1. DVF indicates divalent-free.
≈55%) and neointimal VSMC (by ≈48%) from injured carotids 14 day postinjury (Figure 7a–7d). The I/V relationships of whole-cell LRC currents from different experimental conditions are shown in Figure 7e and 7f, and summary of data is shown in Figure 7g; statistical analyses of data (mean±range; n) are shown in Table 1.

On vascular injury, increase in neointimal thickening was evident in injured vessels 14 days postinjury compared with normal control vessel from sham-operated rats (Figure 7h). Importantly, Orai3 in vivo knockdown caused a significant reduction in neointima size compared with shNT control as observed on hematoxylin-eosin sections (Figure 7h). Both the neointimal size (N) and the ratio of neointima/media (N/M) were reduced on Orai3 knockdown compared with control (Figure 7i and 7j).

Discussion

Our data reveal a store-independent mechanism of Orai channel activation via PLC-coupled receptors and support a model whereby Orai1/3 channel activation by thrombin requires cytosolic LTC4 produced on thrombin receptor ligation. We show that these LTC4-regulated Ca2+ channels do not require store depletion for activation. We also show that unlike PDGF, thrombin does not cause sustained store depletion. The fact that thrombin does not deplete stores, despite the presence of a Ca2+ spike in nominally Ca2+-free solutions, is likely attributable to transient IP3 production because the protease-activated receptor 1, which is the major thrombin receptor in VSMC, is used once and then discarded. However, in other cell types, such as fibroblasts and endothelial cells, signaling by thrombin is maintained by delivery of new protease-activated receptors to the PM from a preformed intracellular pool.37 Interestingly, previous data in cultured endothelial cells showed that thrombin activates SOCE and CRAC currents that are dependent on store depletion and classical STIM1/Orai1 pathway.38 The implications of all these results are that not only do different agonists activate distinct Ca2+ entry pathways in a given cell type but also the same agonist could activate a different Ca2+ entry route, depending on the cell type in question. Our work introduces a novel paradigm, whereby specific PLC-coupled agonists activate specific Ca2+-selective entry pathways in the same cells. This implies that PM receptors ensure the specificity of the Ca2+-selective channel activated and likely the downstream Ca2+-responsive transcription factors and corresponding physiological functions. This diversity of Ca2+-selective conductances through Orai subunit heteromultimerization and alternative activation mechanisms would presumably

Figure 6. Orai3 and leukotrieneC4 (LTC4)-regulated calcium (LRC) currents are upregulated in vascular smooth muscle cell (VSMC) after vascular injury. Whole-cell patch-clamp electrophysiological recordings on VSMCs freshly isolated from media of noninjured carotids (A, n=4) or from either media or neointima of injured carotid arteries. Dialysis of LTC4 through the patch pipette activated Ca2+-selective LRC currents only in VSMC isolated from either media (B) or neointima (C) of injured vessels 14 days postinjury. Na+ current/voltage (I/V) relationships are taken from data points which are indicated by asterisks and shown in D. Statistical summary for this experiment is also shown in (E). Lentiviral infection with shRNA targeting Orai3 (shOrai3) after balloon injury prevented upregulation of Orai3 in injured carotid artery with no significant effect on Orai1 (F–H; n=5). Control nontargeting shRNA (shNT) and Orai3 shRNA (shOrai3) lentiviruses efficiently infected carotid arteries as evidenced by green fluorescent protein (GFP) expression in the protein lysate of media and neointima from left (injured) carotid arteries 14 days after injury and infection (F); no GFP signal was detected in the protein lysate from the right (noninjured and noninfected) carotid artery. DVF indicates divalent-free.
help increase the repertoire of spatial cellular Ca\textsuperscript{2+} microdomains for the purpose of selective Ca\textsuperscript{2+} signaling in complex mammalian organisms.

Thrombin has been shown to have multiple pleiotropic effects: it impacts on VSMC contractility and proliferation and is a major contributor to vascular remodeling. Thrombin is produced massively after vascular injury and during development of atherosclerosis.\textsuperscript{39} The thrombin receptors protease-activated receptors are also upregulated in VSMCs during injury. In this study, we showed that Orai3, the unique component of thrombin-activated Orai1/3 channels, is upregulated in synthetic VSMCs in vitro and in medial and neointimal VSMCs in vivo in a rat model of vascular injury. We also show that preventing Orai3 upregulation during carotid injury using lentiviral particles encoding shRNA reverses the increase in Orai3 protein levels 14 days postinjury, inhibits LTC\textsubscript{4}-regulated calcium channel activation in medial and neointimal VSMC, and inhibits neointima formation. We and others previously showed that STIM1 and Orai1 are important mediators in neointima formation.\textsuperscript{16,26–29} Orai3 or Orai3-containing channels could represent a potential target for treatment of VSMC remodeling during vascular occlusive diseases and might represent a better target than Orai1 or STIM1 because STIM1/Orai1-mediated CRAC is ubiquitous and prominently functional in many tissues, including immune cells and skeletal muscle; the major defects in Orai1-deficient patients and mice are severe immunodeficiency and skeletal muscle hypotonia.\textsuperscript{40}
We show that STIM1 is required downstream of LTC4 action during the activation of this novel LTC4-regulated calcium channel. This fact, along with the high Ca2+ selectivity of this channel, suggests that STIM1 might be a component of the Orai1/3 channel complex. Indeed, recent data from the Prakriya group showed that STIM1 endows, otherwise non-selective, Orai Ca2+ channels with high Ca2+ selectivity.41 Several questions remain to be answered by future structural studies as follows: How LTC4 triggers Orai1/3 channel activation and whether this is a direct action? If it is through direct action, what are the domains in STIM/Orai that are involved in this interaction? What is the exact stoichiometry of LTC4-activated channels in VSMC? The answer to these questions and others will likely help in the targeting of this channel for the purpose of therapy.

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Disclosures
None.

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**Novelty and Significance**

**What Is Known?**

- The ubiquitous store-operated Orai1 Ca\(^{2+}\) channels show low expression in healthy quiescent vascular smooth muscle cells (VSMC), but their expression is increased during VSMC remodeling in a proliferative migratory phenotype.
- Activation of VSMC with the platelet-derived growth factor, a VSMC mitogen, activates Orai1-mediated Ca\(^{2+}\) entry through a mechanism involving endoplasmic reticulum Ca\(^{2+}\) store depletion and subsequent interaction of the Ca\(^{2+}\) sensor stromal interacting molecule1 with Orai1.
- Molecular knockdown of Orai1 inhibits neointima formation in response to balloon injury in rat carotid arteries.

**What New Information Does This Article Contribute?**

- Knockdown of Orai3 in balloon-injured carotid arteries using lentivirus-encoding shRNA prevents Orai3 upregulation, inhibits LTC\(_{4}\)-activated currents, and decreases neointima formation.
- Orai1, a store-operated Ca\(^{2+}\) channel activated by ligation of phospholipase C-coupled receptors, is required for neointima formation on vascular injury. However, Orai1 is functional in many cell types and tissues, which could complicate its use as a specific target in VSMC-related pathologies. Orai1 has 2 homologs Orai2 and Orai3; Orai2 is expressed exclusively in vertebrates, whereas Orai3 is expressed exclusively in mammals. The role of Orai2 and Orai3 in the vascular system remained unknown. We describe a new role of Orai3 in VSMC Ca\(^{2+}\) signaling and remodeling. We show that Orai3 contributes to a novel heteromeric Orai1/3 Ca\(^{2+}\) entry channel in thrombin-activated VSMC. We found that Orai1/3 channel activation is store independent and mediated by cytosolic LTC\(_{4}\) produced downstream of thrombin receptor stimulation. Furthermore, Orai3 expression and LTC\(_{4}\)-activated channel activity increase in VSMC on rat carotid artery injury, whereas knockdown of Orai3 in injured carotids inhibits Orai3 upregulation, LTC\(_{4}\)-activated channels, and neointima formation. These findings suggest that Orai3 represents a novel drug target for controlling VSMC remodeling during vascular injury or disease, and that Orai3 may be a better target than the ubiquitous Orai1 channel.
Store-Independent Orai1/3 Channels Activated by Intracrine Leukotriene C4: Role in Neointimal Hyperplasia

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Store-Independent Orai1/3 Channels Activated by Intracrine Leukotriene C4: Role in Neointimal Hyperplasia

By
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ONLINE METHODS

MATERIALS
Recombinant rat PDGF-BB from R&D Biosystems; Thapsigargin was purchased from Calbiochem; Arachidonic acid from Krackeler Scientific; Leukotriene C4, Leukotriene B4 and Leukotriene C4 ELISA kit from Cayman Chemical; heparin and thrombin from Sigma; 1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA, Cs+ salt) was from Invitrogen. All siRNA sequences were obtained from Dharmacon; shRNA against LTC4S (shLTC4S), Orai3 (shOrai3) and a control non-targeting shRNA (shNT) all cloned in the pGipz vector were purchased from Open Biosystems. ShRNA against the fly luciferase (shLuc), STIM1 (shSTIM1) and Orai1 (shOrai1) described earlier(1) were cloned in our laboratory in the pFUGW vector (see sequences in Online Table III). Specific primers for rat STIM, Orai, and TRPC are listed in Online Table III. Anti-STIM1 antibody was purchased from BD Biosciences, anti-β-actin NH2-terminal domain from Sigma, and anti-Orai1 (extracellular; catalog no. ACC-060) from Alomone, anti-Orai3 (CT) from Prosci Inc. All other chemical products were obtained from Fisher Scientific unless specified otherwise.

METHODS

VSMC dispersion and culture
The use of rats for these experiments has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the Albany Medical College Animal Resource Facility, which is licensed by the US Department of Agriculture and the Division of Laboratories and Research of the New York State Department of Public Health and is accredited by the American Association for the Accreditation of Laboratory Animal Care. Male adult rats (150 g) were euthanized by suffocation in a CO2 chamber. Aortas or carotids were dissected out into ice-cold physiological saline solution. Fat tissues and endothelium were removed. The artery was cut into small pieces and digested with a papain solution for 20 min at 37°C and then with a mixture of collagenase II and collagenase type H for 15 min at 37°C. The digestion solution was removed, and the cells were washed and gently liberated with a fire-polished glass pipette and transferred to culture plates. Isolated cells are routinely tested for expression of VSMC markers (SM22α) using immunofluorescence. Isolated VSMCs undergo phenotypic modulation in culture that is complete within 30 hours. These synthetic proliferative VSMCs that are reminiscent of vascular disease conditions are maintained in culture (45% DMEM-45% Ham's F-12–10% FBS supplemented with L-glutamine) at 37°C, 5% CO2, and 100% humidity, passaged (synthetic), and used in all experiments up to passage 8. For freshly isolated VSMCs, dispersed cells are used seeded immediately after isolation for patch clamp experiments or lysed for Western blots.
Cell transfections
Sets of four different siRNAs per target gene were initially assessed for their ability to reduce mRNA levels using quantitative RT-PCR (qPCR) as described below; the primers used for each target mRNA are listed in Online Table III. SiRNA sequences that induced significant decreases in their target mRNA (over 80%) without cross-effects on other mRNAs were used in Western blotting to confirm protein knockdown as described below. All transfections in VSMCs were done using the Nucleofector device II (Amaza Biosystems, Gaithersburg, MD) using program # D33 according to the manufacturer’s instructions. 0.5 µg of green fluorescent protein (GFP) was co-transfected with siRNA for identification of successfully transfected cells. The control siRNA is a scrambled siRNA sequence.

Ca²⁺ measurements
Ca²⁺ was measured as described previously(1-4). Briefly, coverslips with attached cells were mounted in a Teflon chamber and incubated at 37°C for 1 hour in culture medium (DMEM with 10% FBS) containing 4 µM Fura 2-AM (Molecular Probes, Eugene, OR). Cells were then washed and bathed in HEPES-buffered saline solution (in mM: 140 NaCl, 1.13 MgCl₂, 4.7 KCl, 2 CaCl₂, 10 D-glucose, and 10 HEPES, with pH adjusted to 7.4 with NaOH) for ≥10 min before Ca²⁺ was measured. For Ca²⁺ measurements, fluorescence images of several cells were recorded and analyzed with a digital fluorescence imaging system (InCyt Im2, Intracellular Imaging; Cincinnati, OH). Fura 2 fluorescence at an emission wavelength of 510 nm was induced by exciting alternately at 340 and 380 nm. The ratio of fluorescence at 340 nm to that at 380 nm was obtained on a pixel-by-pixel basis. All experiments were conducted at room temperature.

Whole-cell patch clamp recordings
Conventional whole-cell patch-clamp recordings were carried out using an Axopatch 200B and Digidata 1440A (Axon Instruments, NY) as previously published with few important modifications (2, 4, 5). To reduce the noise to a minimum, we added in series a humbug® noise eliminator that eliminates electrical interference such as simple 50/60Hz sine waves, mixtures of 50/60Hz harmonics, noise spikes from dimmers and complex noise from fluorescent lamps. All experiments were performed at room temperature (20-25°C). Pipettes were pulled from borosilicate glass capillaries (World Precision Instruments, Inc., Sarasota, FL) with a P-97 flaming/brown micropipette puller (Sutter Instrument Company, Novatao, CA) and polished with DMF1000 (World Precision Instruments). Resistances of filled glass pipettes were 2–3 MΩ. Series resistances were in the range of 2–10 MΩ. The liquid-junction potential offset was around 4.6 mV and was corrected. Only cells with tight seals (>16 GΩ) were selected for break in. Immediately after establishing the whole-cell patch-clamp configuration, we start the recording by running a 250 ms voltage ramp (from +100 mV to –140 mV) every 2 s and performing a first DVF pulse before current development. These first I/V curves obtained in Ca²⁺-containing bath solutions (position 1 in scheme) and divalent free (DVF) bath solutions (position 2) represent background currents that are subtracted from agonist-activated Ca²⁺ currents (position 3) and Na⁺ currents (obtained in DVF bath solutions; position 4), respectively. After the currents are fully activated by different stimuli, I/V curves are obtained for Ca²⁺ currents (in Ca²⁺-containing bath solutions) and Na⁺ currents (in
DVF bath solutions. Using Origin Lab 7.5 software (OriginLab, Northampton, MA, USA), I/V curves corresponding to background currents obtained in Ca\(^{2+}\) and Na\(^+\) are subtracted from the I/V curves obtained in Ca\(^{2+}\) and Na\(^+\) after stimulus/agonist addition and maximal current activation. Namely, for Ca\(^{2+}\) currents (curve 3 – curve 1) and Na\(^+\) currents (curve 4 – curve 2), respectively. The subtracted I/V curves are represented as independent I/V curves in all figures. Cells were maintained at a 0 mV holding potential during experiments. Reverse ramps were designed to inhibit Na\(^+\) channels potentially expressed in VSMCs. High MgCl\(_2\) (8mM) was included in the patch pipette to inhibit TRPM7 currents, and 3μM nimodipine was added to the bath solution to generally stabilize membrane patches and reach better seals.

**Solutions employed for whole cell patch clamp electrophysiology**

**Thrombin- and AA-activated currents**

**Bath Solution:** 135mM Na-methanesulfonate, 10mM CsCl, 1.2mM MgSO\(_4\), 10mM HEPES, 20mM CaCl\(_2\), and 10mM glucose (pH was adjusted to 7.4 with NaOH). 100nM Thrombin or 8μM AA was added to the bath where indicated in figures.

**Pipette Solution:** 145mM Cs-methanesulfonate, 10mM Cs-1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (Cs-BAPTA), 5mM CaCl\(_2\), 8mM MgCl\(_2\), and 10mM HEPES (pH adjusted to 7.2 with CsOH). Calculated free Ca\(^{2+}\) is 150nM as estimated using Maxchelator software (http://maxchelator.stanford.edu/).

**Store depletion-activated currents**

**Bath Solution:** 135mM Na-methanesulfonate, 10mM CsCl, 1.2mM MgSO\(_4\), 10mM HEPES, 20mM CaCl\(_2\), and 10mM glucose (pH was adjusted to 7.4 with NaOH).

**Pipette Solution:** 145mM Cs-methanesulfonate, 20mM Cs-1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (Cs-BAPTA), 8mM MgCl\(_2\), and 10mM HEPES (pH adjusted to 7.2 with CsOH).

**Leukotriene\textsubscript{C4}**-activated currents

**Bath Solution:** 135mM Na-methanesulfonate, 10mM CsCl, 1.2mM MgSO\(_4\), 10mM HEPES, 20mM CaCl\(_2\), and 10mM glucose (pH was adjusted to 7.4 with NaOH).

**Pipette Solution:** 145mM Cs-methanesulfonate, 10mM Cs-1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (Cs-BAPTA), 5mM CaCl\(_2\), 8mM MgCl\(_2\), and 10mM HEPES (pH adjusted to 7.2 with CsOH). Calculated free Ca\(^{2+}\) was 150nM using Maxchelator software (http://maxchelator.stanford.edu/). 100nM Leukotriene\textsubscript{C4} (LTC\(_4\)) was added to the pipette solution when indicated.

**Divalent-free (DVF) bath solution:** 155mM Na-methanesulfonate, 10mM HEDTA, 1mM EDTA, and 10mM HEPES (pH 7.4, adjusted with NaOH).

**Western blotting**

Cells were lysed using RIPA lysis buffer (50mM Tris-HCl (pH 8.0), 150mM NaCl, 1% Triton X-100, 0.2mM EDTA, 0.1% SDS, 0.5% Sodium deoxycholate, 2mM phenylmethylsulfonyl fluoride (PMSF), 10% protease inhibitor cocktail (Roche), 10% phosphatase inhibitor cocktail (Roche). Protein concentrations were determined and proteins (20–100μg) in denaturing conditions were subjected to SDS-PAGE (8%-14%) and then electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad). After the blots were blocked with 5% nonfat dry milk (NFDM) dissolved in Tris-buffered saline containing 0.1% Tween 20 (TTBS) either overnight or 2 h at room temperature, they were washed three times with TTBS for 5 min each and incubated overnight at 4°C with specific primary antibodies [anti-STIM1 (BD Biosciences, 1:250 dilution), anti-Orai1 (Alomone; 1:1,000 dilution), anti-Orai3 (ProSci CT, 1: 125 dilution), anti-GFP (Abcam, 1:1000 dilution), anti-LTC\(_4\)S (sc-20108, Santa Cruz Biotechnology, Inc.; 1:500) and β-actin NH\(_2\)-terminal
(NT) domain (Sigma; 1:35000 dilution) in TTBS containing either 1% BSA or 2% NFDM. On the next day, membranes were washed (3 times for 5 min each) with TTBS and incubated for 1h at room temperature with horseradish peroxidase-conjugated secondary antibodies [anti-mouse antibody (1:10,000 dilution; Jackson) or anti-rabbit IgG (1:20,000 dilution; Jackson) in TTBS containing 2% NFDM. Protein bands were visualized by enhanced chemiluminescence using Super Signal West Pico or Femto reagents (Pierce). Signal intensity was measured with a Fuji LAS4000 Imaging Station. Membranes were then stripped and reprobed with β-actin antibody to verify equal loading and densitometric analysis was performed using Image J software.

FRET experiments
VSMCs were transfected by electroporation with the Cameleon D1ER sensor (3μg plasmid DNA per 10⁶ cells) and seeded on glass-bottom petri dishes (MatTek) for live confocal imaging using a Zeiss LSM 510 META confocal microscope as described above. For these experiments the Cameleon D1ER sensor (kindly provided by Dr. Roger Y. Tsien, University of California San Diego) was excited with the 458 nm line of the argon laser and the emitted fluorescence was filtered with band filters for the CFP channel (458-490) and the YFP channel (511-554), respectively. ImageJ was employed to subdivide the cell into regions of interest (ROIs) for data quantification. The FRET/CFP ratio is reported as net FRET value. All experiments were conducted at room temperature.

LTC₄ measurement using ELISA
Leukotriene C₄ (LTC₄) concentrations were measured from VSMC culture supernatants before (t=0) and after stimulation (5min or 15 min) with maximal concentrations of thrombin. The assay was performed using the competitive LTC₄ Enzyme Immunoassay Kit obtained from Cayman Chemicals (#520211).
In brief, VSMCs were seeded on 6 well plates (50 x 10³ cells/well) to 50% confluence, and allowed to recover for 48 hours. Before the assay, cells were washed 3 times with 500μL HBSS and stabilized in the incubator (37°C and 5% CO₂) for 5 minutes. HBSS was exchanged with a HBSS solution containing 500nM thrombin and incubated for 5 and 15 minutes, respectively. The supernatant from 3 wells representing un-stimulated cells and cells stimulated for different time points (5 or 15 min) were collected and kept on ice. Three wells for each condition were assayed in triplicates essentially according to the manufacturer’s protocol. Absorbance reading on plates was performed in a microplate reader at a wavelength of 405 nm. Raw absorbance readings of known standards (provided in kit) and unknown experimental data points were analyzed using a 4-parameter logistic fit. This analysis was achieved by employing a computer spreadsheet for data analysis provided by the manufacturer and available online at www.caymanchem.com/analysis/eia. LTC₄ concentrations were calculated in pg/mL and statistical analyses were performed as described below. Data shown are representative of three independent experiments performed on different days.

Production of lentiviral particles
Lentiviral particles encoding rat specific shRNA against STIM1, Orai1 and Luciferase control (cloned in the viral vector pFUGW-GFP3) and shRNA against Orai3, LTC₄S, and a non-targeting control shRNA (cloned in the lentiviral vector pGipz-GFP) were produced using standard protocols. The shRNA sequences targeting STIM1, Orai1 and Luciferase were previously described (1). Sequences or origin of shRNAs are included in Online Table III. Viral particles were generated using standard protocols. Briefly, PolyJet was used as a transfection reagent (SignaGen) to transfect HEK293FT cells (Invitrogen). The lentiviral constructs pCMV-VSVG, pCMV-dR8.2 and either pFUGW or pGipz encoding-shRNA were co-transfected into a flask of 95% confluent HEK293FT cells. Cell culture media with viral particles were collected at 48h and 72h after transfection and were concentrated using Amicon Ultra-15 filter by
These viral particles were then employed to induce efficient knockdown of protein of interest either in vivo or in vitro. For in vitro infections, cells were studied 7 days post-infection; we determined that at this time point, protein knockdown was optimal.

**Balloon injury of rat carotid arteries**
The use of rats for these experiments has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the Albany Medical College Animal Resource Facility, which is licensed by the US Department of Agriculture and the Division of Laboratories and Research of the New York State Department of Public Health and is accredited by the American Association for the Accreditation of Laboratory Animal Care. Male Sprague-Dawley rats (350–400 g body weight; Taconic Farms, Germantown, NY) were anesthetized with xylazine (5 mg/kg) and ketamine (70 mg/kg) via intraperitoneal injection, and balloon angioplasty was carried out essentially as previously described (1). Briefly, a 2-F Fogarty balloon was inserted through a small arteriotomy in the external carotid artery and passed into the common carotid artery. After balloon inflation to a pressure of 1.5 atm, the catheter was partially withdrawn till the bifurcation of carotid artery and the balloon was deflated. Then the balloon catheter was reinserted and the withdrawal of inflated balloon was repeated two more times. After recovery from operation and anesthesia, animals received a postoperative dose of the analgesic buprenorphine (Buprenex; 0.02 mg/kg s.c.). Sham-operated animals were subjected to a similar surgical procedure with the exception of catheter and balloon insertion.

**Sections, Hematoxylin/Eosin (H&E) staining**
Rats were euthanized at different time points by asphyxiation in a CO₂ chamber and pieces of carotid arteries were isolated and placed in a cryoprotective embedding medium OCT and then snap frozen in liquid nitrogen. The specimens were stored at -80°C or sectioned in a Leica CM3050 cryostat. Sections were treated by pre-cooled acetone for 10 minutes at 4°C and air dried. Hematoxylin/Eosin (H&E) staining was performed following standard protocols (1), with the exception that incubation time of Hematoxylin was increased to 3 minutes and the time with Eosin-Y was decreased to 15 seconds.

**RT-PCR and real-time PCR**
Total RNA was extracted from cells using a Qiagen RNeasy Mini Kit following the manufacturer’s protocol. cDNA was made from 0.5 μg of RNA reverse transcribed using oligo(dT) primers (Invitrogen, Carlsbad, CA, USA) and SuperScript III reverse transcriptase (Invitrogen). PCR reactions were completed using Illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The sense and antisense primers targeting rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), TRPC, STIM, and Orai isoforms are described in Online Table III. All primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). The PCR amplification was done using a MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Amplification started with initial denaturation at 94°C for 5 min and then 40 cycles of denaturation at 94°C for 30 s, annealing at 54.3°C for 1 min, and extension at 72°C for 2 min. Gel electrophoresis was used to identify the PCR products in a 1% agarose gel using ethidium bromide staining. Real-time PCR analysis was performed using a Bio-Rad iCycler and iCycler IQ Optical System Software (Bio-Rad Laboratories). PCR reactions were performed using Bio-Rad iQ SYBR Green Supermix. The PCR protocol started with 5 min at 94°C, followed by 45 cycles of 30 s at 94°C, 30 s at 54.3°C, and 45 s at 72°C. Quantification was measured as sample fluorescence crossed a predetermined threshold value that was just above the background. Expressions of STIM and Orai isoforms were compared to those of the housekeeping gene GAPDH and were measured using comparative threshold cycle values.
Statistical analysis
For patch clamp and animal studies where the n number is less than 10, data are expressed as mean ± range (instead of SEM), and statistical analyses comparing two experimental groups were performed using two-tail t-test with Origin 7.5 software (OriginLab, Northampton, MA). Throughout the figures *, ** and *** indicates p values < 0.05, 0.01 and 0.001 respectively. Differences were considered significant when P < 0.05. Mean ± range and n values are reported in Table 1 for patch clamp data; for animal studies the n number (n=5 for each animal group) is mentioned in the legend to Figure 7. For studies with bigger sample size, including Ca²⁺ imaging, data are represented as mean ± SEM. The n number in the representative Ca²⁺ imaging traces represent the number of cells analyzed simultaneously in the same coverslip and averaged. The two numbers between parentheses (x,y) next to each data point in bar graphs represent: x= number of independent experiments and y= total number of cells from independent experiments/transfections. For comparison between control and one experimental condition two-tail t-test was used. For multiple comparisons, one way ANOVA was performed. The exact p values for all comparisons are included in Table I for all patch clamp experiments and also listed for all the remaining experiments in Online Table II.

Online Table I. Reversal potentials for Ca²⁺ and Na⁺ currents activated by thrombin, LTC₄ and store depletion (20 mM BAPTA) are represented as mean± range.

<table>
<thead>
<tr>
<th>Reversal Potential</th>
<th>I[Ca²⁺]</th>
<th>I[Na⁺]</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin</td>
<td>63.19 ± 12.66</td>
<td>61.96 ± 12.91</td>
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<tr>
<td>LTC₄</td>
<td>60.74 ± 15.96</td>
<td>59.90 ± 15.20</td>
<td>5</td>
</tr>
<tr>
<td>Store depletion (BAPTA)</td>
<td>63.24 ± 18.11</td>
<td>61.89 ± 17.62</td>
<td>7</td>
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</tbody>
</table>

Online Table II. p values for statistical analysis performed on data represented in figures.

<table>
<thead>
<tr>
<th>Figure</th>
<th>P-Value</th>
<th>Figure</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-d</td>
<td>0.00806 (shOrai1 vs shLuc)</td>
<td>3-g</td>
<td>0.0035</td>
</tr>
<tr>
<td></td>
<td>0.00029 (shOrai3 vs shNT)</td>
<td>5-f</td>
<td>0.18276</td>
</tr>
<tr>
<td></td>
<td>0.00849 (shSTIM1 vs shLuc)</td>
<td>5-h</td>
<td>0.00493</td>
</tr>
<tr>
<td></td>
<td>0.00442 (Orai1-E106Q vs Control)</td>
<td>6-g</td>
<td>0.00143</td>
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<tr>
<td></td>
<td>0.00378 (Orai3-E81Q vs Control)</td>
<td>6-h</td>
<td>0.48185</td>
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<tr>
<td></td>
<td>0.00192 (siOrai3 vs siControl)</td>
<td>7-j</td>
<td>0.00002</td>
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<tr>
<td></td>
<td>0.00160 (siSTIM1 vs siControl)</td>
<td>S1-d</td>
<td>0.00435</td>
</tr>
<tr>
<td>3-d</td>
<td>0 (5’)</td>
<td>S2-a</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0 (15’)</td>
<td>S2-d</td>
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</table>
Online Table III. List of primers used for qPCR/PCR; siRNA and shRNA sequences used to target different genes used in the study are also shown.

### PCR Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (3'-5')</th>
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<tr>
<td>rOrai1</td>
<td>ACGTCCACAACCTCAACTCC</td>
<td>ACTGTCGGTCCGTCTTATGG</td>
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<td>rOrai2</td>
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### siRNA Sequences

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<tr>
<td>rOrai1#2</td>
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<td>rOrai2#1</td>
<td>GCCACAACCGUGAGAUCGA</td>
</tr>
<tr>
<td>rOrai2#2</td>
<td>GCAUGCACCAGUACAUCGA</td>
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<tr>
<td>rOrai3#1</td>
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<td>rOrai3#2</td>
<td>GUUUAUGCCCUUUGCCCUA</td>
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<tr>
<td>rSTIM1#1</td>
<td>UAAGGGAAGACCUCAAUU</td>
</tr>
<tr>
<td>rSTIM1#2</td>
<td>CAUCAGAAGUGUAUACUG</td>
</tr>
<tr>
<td>rLTC4S#1</td>
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<tr>
<td>rLTC4S#2</td>
<td>Dharmacon ON-TARGETplus Set of 4 Clones</td>
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<tr>
<td>rTRPC1#1</td>
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<tr>
<td>rTRPC1#2</td>
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</tr>
<tr>
<td>rTRPC4#1</td>
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<tr>
<td>rTRPC6#1</td>
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shRNA Sequences

<table>
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<td>shLuciferase</td>
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<td>shOrai1</td>
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<td>shOrai3</td>
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<td>shSTIM1</td>
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<td>shLTC4S</td>
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ONLINE FIGURE LEGENDS

Online Figure I. Thrombin stimulation does not cause sustained Ca\(^{2+}\) store depletion.

Ca\(^{2+}\) imaging experiments showing that maximal concentrations of PDGF (500 ng/mL) in nominally Ca\(^{2+}\) free media induce Ca\(^{2+}\) release. PDGF was kept throughout and Ca\(^{2+}\) was restored to the extracellular space for 6 min to prevent excessive Ca\(^{2+}\) leak from ER. Subsequent stimulation with maximal concentrations of thrombin (500 nM) in nominally Ca\(^{2+}\) free media failed to cause Ca\(^{2+}\) release from internal stores suggesting that IP\(_3\)-sensitive stores have been completely depleted by PDGF (a). When the order of agonist addition was reversed (i.e. thrombin first), the peak of Ca\(^{2+}\) release induced by PDGF added after thrombin was similar to the peak generated when PDGF was added first (c), suggesting that thrombin only reversibly mobilizes internal Ca\(^{2+}\) stores without causing sustained depletion. Please also note that the Ca\(^{2+}\) release peak induced by thrombin is rather “skinny” when compared to that of PDGF (duration 1 min versus 6 min for PDGF). Statistical analysis on PDGF-induced intracellular Ca\(^{2+}\) release; whether PDGF was used as 1\(^{st}\) or 2\(^{nd}\) agonist, a similar extent of Ca\(^{2+}\) release was observed in both cases (b). However, PDGF-induced Ca\(^{2+}\) release essentially eliminated the ability of subsequently added thrombin to cause intracellular Ca\(^{2+}\) release (d). (x,y) next to each data bar: x= number of independent runs, y= total number of cells. e; ER-Ca\(^{2+}\) levels were measured by the ER-targeted FRET sensor Cameleon-D1ER, upon stimulation with maximal concentrations of thapsigargin (TG; 4 μM), PDGF (500 ng/mL) and thrombin (Th; 500 nM).

Online Figure II. Orai3 knockdown abrogates thrombin-activated Ca\(^{2+}\) entry; thrombin and AA-activated currents are not additive.

a; Because of lack of specific antibody against rat Orai2 protein, we performed quantitative RT-PCR to document specific Orai2 knockdown upon transfection of VSMCs with specific siRNA against rat Orai2 (no effect was seen on Orai1 or Orai3 expression; statistical analysis on data are from three independent transfections, each performed in triplicates). b; Western blot analysis validated the specificity of a second siRNA sequence targeting Orai3; Orai3 protein knockdown was readily detected with no effects on Orai1 or STIM1 proteins. Representative Ca\(^{2+}\) imaging traces from VSMCs transected with the same siRNA Orai3 sequence confirmed
the requirement for Orai3 in thrombin-activated Ca\(^{2+}\) entry (c). Statistical summary of Ca\(^{2+}\) imaging data is shown (d). e-g; Whole-cell patch clamp electrophysiological recordings showing no additivity between thrombin (100nM) and arachidonic acid (AA; 8μM). Na\(^+\) I/V curves and statistical analysis are shown in f and g, respectively.

**Online Figure III. LTC\(_4\) Synthase (LTC\(_4\)S) is required for thrombin-activated Ca\(^{2+}\) entry.**

Whole-cell patch clamp electrophysiological recordings testing for additivity between LT\(_4\) and thrombin (a-c), d; addition of LT\(_4\) to the bath solution failed to activate Ca\(^{2+}\) or Na\(^+\) currents; Na\(^+\) I/V relationship is shown in e, f; Representative Ca\(^{2+}\) imaging traces (and statistics; g) assessing SOCE activated by thapsigargin in VSMCs transfected with control siRNA or siRNA against LTC\(_4\)S. Note that knockdown of LTC\(_4\)S had no effect on thapsigargin-activated SOCE (f, g). Representative whole-cell patch clamp traces showing thrombin-activated Ca\(^{2+}\) and Na\(^+\) currents in VSMCs transfected with either siRNA control or siRNA targeting LTC\(_4\)S (h-k). I/V relationships show significant inhibition of thrombin-activated Na\(^+\) (j) currents. I/V curves for Na\(^+\) currents are taken from current traces where indicated by color-coded asterisks. Statistical analyses on normalized Na\(^+\) currents are shown in k. Whole-cell patch clamp electrophysiology in VSMCs infected with lentivirus carrying either control non targeting shRNA (shNT; a) or shRNA targeting STIM1 (shSTIM1; b). Inclusion of BAPTA in the pipette activates CRAC currents measured in Ca\(^{2+}\)-containing and DVF solutions that were completely abrogated in cells depleted of STIM1. Na\(^+\) I/V relationships are shown in c and statistics on current densities are shown in d.

**Online Figure IV. STIM1 knockdown abrogates CRAC currents activated by store depletion in VSMCs.**

Whole-cell patch clamp recordings of CRAC currents (a, b) activated by passive store depletion with 20mM BAPTA in VSMCs. VSMCs were infected with lentiviruses encoding either control luciferase shRNA (shLuc; a) or shRNA targeting STIM1 (shSTIM1; b). Inclusion of BAPTA in the pipette activates CRAC currents measured in Ca\(^{2+}\)-containing and DVF solutions that were completely abrogated in cells depleted of STIM1. Na\(^+\) I/V relationships are shown in c and statistics on current densities are shown in d.

**Online Figure V. Orai1 knockdown abrogates CRAC currents activated by store depletion in VSMCs while Orai3 does not.**

Whole-cell patch clamp electrophysiology of CRAC currents activated by passive store depletion with 20mM BAPTA in VSMCs. VSMCs were infected with lentivirus carrying either control shRNA against luciferase (shLuc), or shRNA targeting Orai1 (shOrai1). Orai1 knockdown completely abrogated Na\(^+\)/Ca\(^{2+}\) CRAC currents (b) as compared to control (a). Na\(^+\) I/V relationships for CRAC are shown in (c) and statistical analysis is shown in (d). Whole-cell patch clamp electrophysiology of CRAC currents activated by passive store depletion with 20mM BAPTA in VSMCs. VSMCs were infected with lentivirus-encoding either non-targeting control shRNA (shNT; e) or shRNA targeting Orai3 (shOrai3; f). CRAC currents activated by passive store depletion with 20mM BAPTA (e) were not affected by Orai3 knockdown (f). Na\(^+\) I/V relationships show indistinguishable Na\(^+\) CRAC I/V relationships in both shNT and shOrai3 conditions (g). Statistical analysis is shown in (h).

**Online Figure VI. Orai3 is upregulated in synthetic VSMCs and Orai3 shRNA lentiviruses efficiently infect VSMC in vitro.**

a; Western blot analysis showing upregulation of Orai3 protein levels in cultured synthetic VSMCs (reminiscent of disease states) as compared to quiescent VSMCs freshly dispersed from rat aorta (representing the healthy situation). b; Cultured synthetic VSMCs were infected with lentiviruses-encoding either shRNA control (shNT) or shRNA against Orai3 (shOrai3).
Infected cells are visualized by GFP fluorescence. Phase pictures are shown to gauge the infection efficiency, which is essentially 100%.

Online Figure VII. Store depletion activates CRAC currents in freshly isolated medial and neointimal VSMCs from injured carotid arteries but not in medial VSMCs from control carotids.

Whole-cell patch clamp electrophysiological recordings on VSMCs freshly isolated from either control non-injured media (a, n=4) or from media (b, n=5) and neointima (c, n=5) of injured carotid arteries. Only cells from injured carotids showed development of CRAC currents. Upon dialysis of medial and neointimal VSMCs from injured carotids (14 day post injury) with 20mM BAPTA to cause store depletion, CRAC currents were observed. However, non-injured control medial VSMC showed no CRAC currents upon store depletion with BAPTA. Na⁺ I/V relationships are taken from traces where indicated by the color-coded signs and are shown in (d). Statistical summary is also shown in (e).

ONLINE REFERENCES
Online Figure II

(a) Normalized mRNA Levels (using siOrai2)

(b) Western blot analysis of Orai1, Orai3, STIM1, and β-actin.

(c) Time course of F340/F380 ratio following Th (100nM) stimulation.

(d) Changes in F340/F380 ratio normalized.

(e) Effect of Th (100nM) and AA (8μM) on membrane potential (mV).

(f) Sodium current (pA/pF) before and after Th (100nM) and AA (8μM).

(g) Sodium current (pA/pF) following 1st and 2nd stimulation.

* Significant difference

(4, 96) and (4, 68) indicate sample size and associated value.
Online Figure IV

![Graphs and data analysis showing the effect of BAPTA (20mM) on the current levels in different conditions: shLuc and shSTIM1 with and without BAPTA.](image)
Non-injured

20 mM BAPTA in pipette

20 mM BAPTA in pipette

DVF

DVF

0.2 pA/pF

1 min

Injured-media

Injured-neointima

20 mM BAPTA in pipette

20 mM BAPTA in pipette

DVF

DVF

Na^+ current density (pA/pF)

Non-injured

Inj.-Media

Inj.-Neointima

***

***